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Phylogenetic and ecological analyses of soil and sporocarp DNA sequences reveal high diversity and strong habitat partitioning in the boreal ectomycorrhizal genus *Russula* (Russulales; Basidiomycota)

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Summary

- Although critical for the functioning of ecosystems, fungi are poorly known in high-latitude regions. Here, we provide the first genetic diversity assessment of one of the most diverse and abundant ectomycorrhizal genera in Alaska: *Russula*.
- We analyzed internal transcribed spacer rDNA sequences from sporocarps and soil samples using phylogenetic methods, operational taxonomic unit (OTU) delimitations and ordinations to compare species composition in various types of boreal forest.
- The genus *Russula* is highly diverse in Alaska, with at least 42 nonsingleton OTUs (soil) and 50 phylogroups (soil + sporocarp). *Russula* taxa showed strong habitat preference to one of the two major forest types in the sampled regions (black spruce and birch–aspen–white spruce), and some preference for soil horizon.
- Our results show that the vast majority of *Russula* species are present in the soil samples, although some additional taxa are expected to be found with extended sampling. OTU diversity in black spruce forests was only one-third of the diversity observed in mixed upland forests. Our findings suggest that some of the diversity is niche based, especially along host and successional axes, because most OTUs predictably occurred in specific habitats, regardless of geographical location.

Introduction

There are serious concerns among researchers and the public alike related to the future of global biodiversity, particularly because of the threats represented by climate change scenarios and growing human impact. Despite our constantly improving understanding, we still know very little about the true diversity of life, and our lack of knowledge now severely compromises our ability to recognize and to respond intelligently to recent and future environmental changes (Donoghue *et al.*, 2009). Currently, the majority of biodiversity studies and conservation efforts are focused on vascular plants, vertebrates and, to some extent, insects (Elvebakk, 2005), whereas soils remain a relatively unexplored, yet presumably significant, source of biodiversity (Usher *et al.*, 2005; Buée *et al.*, 2009; Geml *et al.*, 2009).

The boreal forest is the largest terrestrial biome, including *c.* 33% of all remaining forests in the world. It covers an area of *c.* 18.5 million square kilometers and forms a continuous northern circumpolar zone (Bonan & Shugart, 1989; McGuire *et al.*, 1995; Bradshaw *et al.*, 2009). Human influences have typically been less severe in boreal zones than in other forested regions, and disturbance dynamics have mostly been driven by natural processes, such as fire (Essen *et al.*, 1992; Engelmark, 1999; Dahlberg, 2002; Bradshaw *et al.*, 2009). However, recent increases in

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the demand for natural resources have stimulated greater perturbation (Bradshaw *et al.*, 2009), whereas recent climatic changes have altered fire regimes and dynamics of species interactions (Chapin *et al.*, 2006) in boreal regions. Because boreal forests contain *c.* 27% of the world's vegetation carbon inventory and 28% of the world's soil carbon inventory (equivalent to 75% of the total atmospheric carbon) (McGuire *et al.*, 1995; Chapin *et al.*, 2006), the effects of climate and land use change on the structure and function of boreal communities have the potential to influence carbon exchange with the atmosphere (Smith & Shugart, 1993; McGuire *et al.*, 2000). Regions underlain by discontinuous permafrost, such as Interior Alaska, are particularly vulnerable to climate change because the degradation of permafrost, induced by higher temperatures and/or disturbance, alters drainage and exposes permafrost organic matter to decomposition (Frolking *et al.*, 1996; McGuire *et al.*, 2000).

Although critical for the functioning of ecosystems, especially as symbionts of plants and recyclers of organic matter, fungi are particularly poorly known in high-latitude regions (Callaghan *et al.*, 2004). Boreal plant communities, such as those found in Interior Alaska, are frequently described as relatively species poor and having simpler patterns than those in more southern biomes (Dahlberg, 2002; Chapin *et al.*, 2006; Hollingsworth *et al.*, 2006). Ectomycorrhizal (ECM) fungi are among the most important and most abundant fungi in boreal ecosystems (Dahlberg, 2002; Taylor *et al.*, 2007), and form associations with all major tree genera found in this region (e.g. *Picea*, *Betula*, *Populus*, *Alnus*, etc.) (Brunner *et al.*, 1992; Molina *et al.*, 1992; Smith & Read, 1997). Such mycorrhizal associations can form on > 95% of tree roots and participate in the nutrient and carbon transfer between soil and the host plant (Smith & Read, 1997).

Traditionally, our knowledge of fungal diversity has been based almost entirely on the collection and taxonomic study of sporocarps. Macrofungi, such as *Russula* and other mushroom-forming fungi, are among the most studied groups of fungi and have the longest history of diversity studies. Nonetheless, even for macrofungi, basic questions about the number of species at a given location or differences in species richness among types of forest have generally remained unanswered because of taxonomic problems and the scarcity of long-term sporocarp-monitoring projects (Lodge *et al.*, 2004). In recent years, DNA-based studies of soil fungal communities have provided valuable insights into the biodiversity and ecology of fungi, and have provided evidence that there is an enormous amount of fungal diversity in soils that is still mostly unknown (Schadt *et al.*, 2003; O'Brien *et al.*, 2005; Buée *et al.*, 2009). Unfortunately, both sporocarp- and soil-based methods have limitations. The production of sporocarps (on which current surveys for long-term monitoring are based) by any particular species is a largely unpredictable phenomenon, and many species produce nearly invisible sporocarps or are asexual. On the other hand, the identification of lineages by DNA profiling is limited by the available reference sequences from known taxa. Moreover, novel and/or previously unsequenced lineages are often known only from their DNA sequences and, without any data on their morphology, their systematic classification is very difficult or impossible. The combined sampling of sporocarps and soil DNA has been shown to give complementary views of biodiversity, with a relatively large number of taxa detected only by one of the two methods (Porter *et al.*, 2008; Geml *et al.*, 2009).

With the exception of the nonmycorrhizal genus *Agaricus* (Geml *et al.*, 2008), the ECM genus *Lactarius* (Geml *et al.*, 2009) and the *Amanita muscaria* species complex (Geml *et al.*, 2006), the species distribution, phylogenetic diversity and molecular ecology of boreal Alaskan fungi are very poorly known. Therefore, by utilizing the complementary strengths of both soil- and sporocarp-based methods, we analyzed the rDNA sequences generated in a high-throughput fashion from both curated sporocarp collections and soil PCR clone libraries to assess the biodiversity of *Russula* in the boreal forests of Interior Alaska. *Russula* Pers. is a very diverse genus, with *c.* 750 described species worldwide (Kirk *et al.*, 2001), and is one of the most speciose and abundant groups of boreal ECM fungi. Based on their sheer abundance and wide distribution, these fungi presumably have great ecological importance as mycorrhizal partners of boreal trees and shrubs in Alaska. For example, by including all fungi in our soil clone libraries, four of the 30 most abundant taxa belong to this genus (Taylor *et al.*, 2010), and there are many species that fruit regularly and abundantly in various forest types in Alaska (J. Geml, pers. obs.). In addition to assessing their biodiversity, we analyzed how assemblages of *Russula* species change among different plant communities.

Materials and Methods

The study region

The Intermontane Boreal Forest ecoregion (Nowacki *et al.*, 2001) of Interior Alaska is bordered by the Alaska Range to the south, the arctic treeline in the Brooks Range to the north and the climatic treeline to the west. This ecoregion represents the westernmost end of the boreal belt spanning the North American continent. Interior Alaska is an area of discontinuous permafrost, *c.* 75–80% of which is underlain by permafrost, with the exception of most south-facing slopes (Osterkamp & Romanovsky, 1999). The climate in this region is strongly continental with low annual precipitation (286 mm on average), extreme temperatures ranging from −60 to 35°C, and snow covering the ground for 6–9 months of the year (Slaughter & Benson, 1986; Hinzman

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et al., 2005). Despite the fact that most of Interior Alaska was not glaciated, there is little morphological development in the soils, most of them being Inceptisols, Entisols, Histosols or Gelisols (Ahrens *et al.*, 2004; Hollingsworth *et al.*, 2006).

The area's forest vegetation consists of a mosaic of different forest types, formed predominantly as a result of slope, aspect, elevation, parent material and succession following disturbance (mostly fire and flooding). Forest types include black spruce (*Picea mariana*) communities on permafrost-dominated north-facing slopes and lowlands, and mixed birch–aspen–white spruce (*Betula neoalaskana*–*Populus tremuloides*–*Picea glauca*) forests on well-drained south-facing slopes (Viereck *et al.*, 1992; Hollingsworth *et al.*, 2006).

Soil samples were taken at sites monitored by the Bonanza Creek Long Term Ecological Research program (BNZ LTER, http://www.lter.uaf.edu/), representing multiple vegetation types and successional stages of forest development in Interior Alaska (Table 1). Among these, the upland mixed birch–aspen–white spruce stands (indicated by UP numbers) are located in the Bonanza Creek Experimental Forest (BCEF), *c.* 20 km southwest of Fairbanks, Alaska. The sampled black spruce types are broadly distributed among multiple sites, including BCEF, the Caribou-Poker Creek Research Watershed (*c.* 40 km northeast of Fairbanks), as well as areas in the vicinity of Delta Junction and Fairbanks, Alaska. Plant community and soil characteristics of these sites have been described in detail by Hollingsworth *et al.* (2006).

Isolates and DNA extraction

We sampled three upland forest (UP) and four black spruce forest (B) subtypes: early-successional (UP1), mid-successional (UP2) and late-successional (UP3) upland forests and acidic, dry (BAD), acidic, wet (BAW), nonacidic, dry (BND) and nonacidic, wet (BNW) black spruce sites. In each subtype, three replicate plots were sampled. In each plot, 50 soil cores, 1.8 cm in diameter and 10–20 cm deep, always containing both organic and mineral horizons, were taken along five parallel, 100-m transects, in such a way that the cores were at least 10 m from each other to minimize the probability of sampling the same genet repeatedly. Samples were separated into organic and mineral horizons. For every 50 m × 100 m plot, the 50 cores sampled as described above were pooled for each horizon, which resulted in two separate DNA extractions per plot. Thus, 18 separate extractions were made for the upland sites and 24 for the black spruce sites. We replicated the entire process in the following year, resulting in a total of 84 DNA extractions.

Soils were deposited in 50-ml Falcon tubes and stored at −80°C until lyophilization. Soils were then ground using a ball mill at −20°C, with 0.8-mm steel ball bearings. Genomic DNA was extracted from 1–5 g of soil from each composite sample using the Mo Bio Powersoil (Carlsbad, CA, USA) kit following the manufacturer's instructions. The soil DNA extracts were normalized to 2.5 ng μl^{-1} after Picogreen (Invitrogen, Carlsbad, CA, USA) quantification. The methods have been partially described in Taylor *et al.* (2007, 2008) and Geml *et al.* (2009).

In addition, 18 sporocarps were selected for molecular work, representing morphological groups from the total of 799 specimens of *Russula* collected by G. A. Laursen from different forested regions of Alaska over a 35-yr period. All collections are publicly available from the University of Washington Herbarium (WTU) at the Burke Museum. A map of the locations for these and other collections has been published previously (Geml *et al.*, 2008). DNA was extracted from small samples of dried specimens using the E-Z 96® Fungal DNA Kit (Omega Bio-tek, Inc., Doraville, GA, USA) or the DNeasy® Plant Mini Kit (QIAGEN, Inc., Valencia, CA, USA).

PCR, DNA sequencing and contig assembly

The entire internal transcribed spacer (ITS) and partial large subunit regions were PCR amplified from sporocarps in reaction mixtures containing 1.75 μl Ultrapure Water (Invitrogen, Carlsbad, CA, USA), 1 μl 10 × Herculase PCR buffer (Stratagene, La Jolla, CA, USA), 0.05 μl of 100 mM dNTP mixture, 25 mM of each dNTP (Applied Biosystems, Foster City, CA, USA), 0.2 μl Herculase DNA polymerase (Stratagene), 2 μl of 1 μM forward primer ITS1F (Gardes & Bruns, 1993) and reverse primer TW13 (Taylor & Bruns, 1999), and 3 μl of template DNA at a concentration of 0.1 ng μl^{-1}. PCRs were performed using the following temperature program: 95°C for 2 min; 25 or 34 cycles of 95°C for 0.5 min, 54°C for 1 min, 72°C for 2 min; and 72°C for 10 min. Soil DNAs were amplified using Amersham ready-to-go beads. For each soil DNA extract, seven replicate PCRs were performed and pooled. To minimize chimera formation, 25 PCR cycles were performed for soil samples. We utilized a molecular tagging strategy to mark PCR products from various sources with DNA tags, which can then be pooled before library sequencing (Taylor *et al.*, 2008). Thus, the identities of samples were preserved whilst processing a feasibly small number of clone libraries. Tagging was achieved through the addition of 10-base extensions at the 5′-end of the TW13 primer. The pooled PCR products were cloned into the pCR®4-TOPO vector from Invitrogen. The resulting PCR libraries were shipped frozen to the Broad Institute of MIT and Harvard, where transformation, plating, colony picking, Templiphi reactions and sequencing were carried out on automated equipment, using M13 forward and reverse external vector primers. We sequenced at least 1536 clones per library.

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Table 1 Habitat, clone library names, plot numbers and locations (with GPS coordinates) for soil samples used in this study

Habitat and clone library	Plot number	Stand age (yr)	Dominant tree species	Location (latitude, longitude)
Early-successional upland mixed forest UP1 (2004) and UP4 (2005)	UP1 A	23–25	Bene: 77% Potr: 23%	Bonanza Creek LTER site, Parks Hwy. (64.73473541; –148.2976791)
	UP1 B	23–25	Bene: 94% Pigl: 0.5% Potr: 5.5%	Bonanza Creek LTER site, Parks Hwy. (64.73195762; –148.2974016)
	UP1 C	23–25	Bene: 3% Poba: 1% Potr: 96%	Bonanza Creek LTER site, Parks Hwy. (64.73195762; –148.2974016)
Mid-successional upland mixed forest UP2 (2004) and UP5 (2005)	UP2 A	93–98	Bene: 51% Pigl: 46% Poba: 3%	Bonanza Creek LTER site, Parks Hwy. (64.69390269; –148.3537914)
	UP2 B	93–98	Bene: 54% Pigl: 22.5% Poba: 12.5% Potr: 11%	Bonanza Creek LTER site, Parks Hwy. (64.68945831; –148.3599027)
	UP2 C	93–98	Bene: 0.5% Pigl: 10.5% Potr: 89%	Bonanza Creek LTER site, Parks Hwy. (64.68862521; –148.3790685)
Late-successional upland mixed forest UP3 (2004) and UP6 (2005)	UP3 A	225–230	Bene: 32% Pigl: 62% Potr: 6%	Bonanza Creek LTER site, Parks Hwy. (64.7666796; –148.2740661)
	UP3 B	225–230	Bene: 39% Pigl: 59.5% Potr: 1.5%	Bonanza Creek LTER site, Parks Hwy. (64.75973477; –148.2446238)
	UP3 C	225–230	Bene: 30% Pigl: 66% Potr: 4%	Bonanza Creek LTER site, Parks Hwy. (64.72445795; –148.324901)
Black spruce, acidic, dry TKN7 (2004) and TKN11 (2005)	TKN-0012	200–210	Bene: 1% Pima: 99%	Washington Creek, Elliott Hwy. (65.16721667; –147.8941333)
	TKN-0122	90–100	Pima: 100%	Delta Junction, Alaskan Hwy. (63.90620584; –145.3711972)
	TKN-0001	95–100	Bene: 1% Pima: 99%	Bonanza Creek LTER site, Parks Hwy. (64.76572442; –148.295527)
Black spruce, acidic, wet TKN8 (2004) and TKN12 (2005)	TKN-0015	170–180	Pima: 100%	Washington Creek, Elliott Hwy. (65.15451667; –147.8631667)
	TKN-0022	150–180	Pima: 100%	Babe Creek, Elliott Hwy. (64.99653333; –147.65305)
	TKN-0109	90–104	Pima: 100%	Caribou Poker Creek Research Watershed, Steese Hwy. (65.16166012; –147.4878514)
Black spruce, nonacidic, dry TKN9 (2004) and TKN13 (2005)	TKN-0039	130–190	Pima: 100%	Ballaine Rd., Fairbanks (64.91003773; –147.8218001)
	TKN-0123	97–100	Pima: 100%	Delta Junction, Alaskan Hwy. (63.92459496; –145.3167362)
	TKN-0126	120–130	Pima: 100%	Delta Junction, Alaskan Hwy. (63.84655372; –145.7208714)
Black spruce, nonacidic, wet TKN10 (2004) and TKN14 (2005)	TKN-0051	68–91	Lala: 3% Pigl: 3% Pima: 94%	UAF Arboretum, Fairbanks (64.86588323; –147.8736745)
	TKN-0119	280–320	Pima: 100%	Delta Junction, Alaskan Hwy. (63.81379184; –144.9532331)
	TKN-0040	179–216	Lala: 3% Pigl: 1% Pima: 96%	Ballaine Rd., Fairbanks (64.9148493; –147.8297945)

For each habitat, three plots were sampled (50 soil cores per plot) and the extracted DNAs were pooled for PCR clone library constructions. This sampling was carried out in 2004 and repeated in 2005. Bene, *Betula neoalaskana*; Lala, *Larix laricina*; Pigl, *Picea glauca*; Pima, *Picea mariana*; Poba, *Populus balsamifera*; Potr, *Populus tremuloides*; UP, upland forest, LTER, long-term ecological research.

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Initial bioinformatic steps

Sequence data obtained for both strands were edited and assembled for each sporocarp or soil clone using Aligner v. 1.3.4 (CodonCode Inc., Dedham, MA, USA). We identified soil clone sequences to genera, based on similarity searches using FASTA (Pearson & Lipman, 1988), against a reference database containing all fungal ITS sequences from GenBank. We proceeded with 4127 soil sequences that were identified as *Russula*. Twenty-six intergeneric chimeric sequences were detected by conducting separate BLAST sequence similarity searches for the ITS1 and ITS2 regions, and were excluded from further analyses. We regarded a sequence as chimeric when it had > 90% sequence similarity to different genera in ITS1 and ITS2. Furthermore, we excluded six additional sequences that were suspected to be intrageneric chimeras (where ITS1 and ITS2 were 100% similar to different species). After collapsing identical sequences, we deposited representative sequences of all ITS types from soil clone libraries and herbarium specimens in Genbank (EU711724–EU712018, EU712020–EU712096, GU981742–GU981745). Of these, sporocarp sequences were: EU711748, EU711763–EU711769, EU711957–EU711959, EU711976, EU711999–EU712001, EU712011–EU712012, EU712092.

Delimitation of operational taxonomic units (OTUs)

We grouped all soil clones into OTUs to reduce the sample size for the phylogenetic analyses and to provide input for ecological comparisons among habitats. Based on previously published fungal diversity studies (e.g. O'Brien *et al.*, 2005; Arnold & Lutzoni, 2007; Higgins *et al.*, 2007; Geml *et al.*, 2009), we used 97% ITS sequence similarity for OTU delimitation. Pairwise sequence similarity-based groupings were estimated by Cap3 (Huang & Madan, 1999). It should be noted that Cap3 uses a single-linkage clustering algorithm, meaning that sequences less than, for example, 97% similar will be grouped together if an intermediate sequence of > 97% similar to both is found. Species accumulation curves and bootstrap estimates of total richness were computed using EstimateS (Version 7.5, R. K. Colwell, http://purl.oclc.org/estimates). Because sequencing errors can overestimate the diversity of 'rare taxa' (e.g. see Kunin *et al.*, 2009), we opted to be conservative and excluded all singletons from the phylogenetic analyses and ordinations, retaining a total of 4091 soil clones in all OTUs. We realize that this may have resulted in the exclusion of some real taxa. However, in our preliminary analyses, some singletons grouped around the distinct OTUs, whereas some were placed on long branches with no close relative identified. Therefore, their contribution to the identification of *Russula* species in Alaska was limited. There was one exception, marked by S1 in Fig. 2, which perfectly matched a sporocarp sequence in our sample (GAL15229) and a *R. pallescens* sequence from GenBank.

Phylogenetic analyses

A randomly chosen clone was generally selected from each resulting OTU for phylogenetic analyses. When an OTU was detected in both major forest types (upland and lowland), we selected one representative clone from each habitat. Sequences derived from sporocarps were included to link soil clones to taxa with voucher specimens. Because our goal was to conduct the most complete phylogenetic diversity assessments possible, all other *Russula* ITS sequences were downloaded from GenBank, including unidentified environmental samples. From these, we selected nonidentical representatives of identified taxa and unidentified sequences that were at least 600 bp long. We constructed a multiple sequence alignment using ClustalW (Thompson *et al.*, 1997) which was subsequently refined by MUSCLE (Edgar, 2004) and finally corrected manually. Phylogenetic analyses were conducted using maximum-likelihood (ML) and Bayesian methods in Garli 0.94 (Zwickl, 2006) and MrBayes (Huelsenbeck & Ronquist, 2001), respectively. The best-fit evolutionary model was determined by comparing different evolutionary models with varying values of base frequencies, substitution types, α-parameter for the γ-distribution of variable sites and proportion of invariable sites via the Akaike Information Criterion (AIC) using PAUP* (Swofford, 2002) and Modeltest 3.06 (Posada & Crandall, 1998). Gaps were scored as 'missing data'. The Life Sciences Informatics cluster portal (http://biotech.inbre.alaska.edu/) was used for all analyses.

Comparing *Russula* communities among vegetation types

We used the 97% ITS sequence similarity OTUs inferred earlier as input data for the various ordination methods. Herbarium sequences were excluded as they were not randomly sampled. To visualize the variation in the occurrence and abundance of *Russula* OTUs across sites and years, we carried out ordination using nonmetric multidimensional scaling (NMS) in PC-Ord (McCune & Grace, 2002). NMS is gaining popularity as an ordination technique in ecology because it relaxes many of the assumptions that plagued earlier methods, such as linear (principal components analysis, PCA) or Gaussian (canonical correspondence analysis, CCA) species responses to environmental gradients (McCune & Grace, 2002). Horizon was included in a site environmental factor matrix as a categorical variable, whereas tree abundances (Alaskan birch, trembling aspen, black + white spruce) and successional stage were described on simple ordinal scales (0, 0–10 trees per 300 m^2; 1, moderate densities of 11–80 trees per 300 m^2;

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2, 81–300 trees per 300 m^2; 0, early succession UP1 sites; 1, mid-succession UP2 sites; 3, late succession UP3 and TKN black spruce sites). Following NMS ordination of sites, we examined the Pearson correlation values between the primary community ordination axes and these environmental variables. We also tested whether *Russula* communities were statistically different across years and habitat types using a multiresponse permutation procedure (MRPP), and examined the responses of individual species to environmental gradients using indicator species analyses, also in PC-Ord. For the indicator species analyses, the ordinal variables described above were reduced to categorical presence/absence groupings. In all of these analyses, OTUs occurring in two or fewer sites were excluded. We first conducted analyses with samples separated by year (2004, 2005) and horizon (organic, mineral) for all sites, and separately for upland and black spruce sites. Because MRPP indicated that *Russula* communities did not differ significantly by year or horizon, the OTU abundances for both years and both horizons for a particular site were combined in subsequent NMS ordinations (Figs 3, 4).

Results

Delimitation of OTUs

We recovered 46 distinct OTUs from the soil sequences based on 97% ITS sequence similarity, four (8.7%) of which were singletons. The mean Chao 1 estimate of the total number of taxa was 46.6 with Chao 1 SD = 1.06. Although bootstrap estimates of species richness (B = 48.75) significantly exceeded the observed species richness ($B > S_{obs}$ + 1 SD; where the observed species richness S_{obs} = 46 and the standard deviation of S_{obs} SD = 0.92), species accumulation curves were nearly saturated (Fig. 1).

Phylogenetic analyses

The ITS alignment contained 306 sequences and 930 characters, 513 of which were parsimony informative. The General-Time-Reversible model, with calculated proportion of invariable sites (I = 0.1307) and estimated α-parameter (0.8644) of γ-distribution (GTR + I + G), was selected as the best-fit evolutionary model. ML analyses resulted in a single tree (Log_e = 29 146.2106) (Fig. 2). Phylogenetic groups were identified as the smallest clades supported by a Bayesian posterior probability ≥ 0.95 (Fig. 2). In a very few instances, phylogroups were paraphyletic with respect to other well-supported phylogroups. When two Alaskan OTUs were sister groups, we treated them as distinct phylogroups, because previous studies in various genera (e.g. Geml *et al.*, 2008, 2009) have shown that 97% OTUs tend to be well-supported clades that are equally or more inclusive than phylogroups as defined above. Phylogroups were named following the OTU identifications in Table 2 or based on closest relatives (sporocarp-only groups). In the few instances in which OTUs were split into different phylogroups, we maintained the original OTU name, but distinguished the phylogroups by numbers (Fig. 2).

We detected 50 phylogroups of *Russula* in the boreal forests of Interior Alaska that were widely distributed on the phylogenetic tree. We were able to identify 30 of them to species, based on publicly available closely related sequences. Twenty phylogroups could not be identified to species. Of these, five represented previously sequenced, but

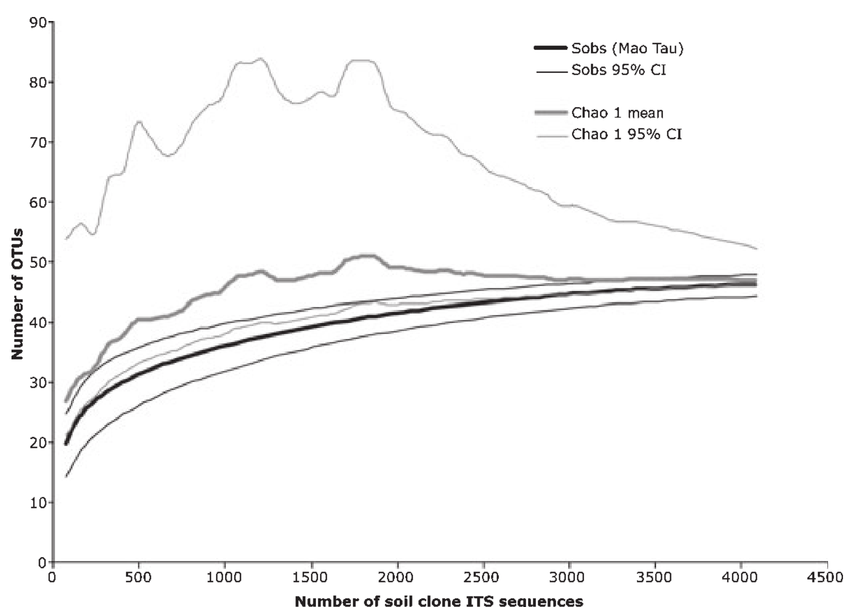

Fig. 1 Species accumulation curves for *Russula* operational taxonomic units (OTUs) in our soil samples based on 97% internal transcribed spacer (ITS) sequence similarity. The observed number of OTUs is given as Mao Tau estimates (S_{obs}), and the estimated total number of OTUs is given as Chao 1 estimates, with 95% confidence intervals (CI).

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unidentified taxa, with close matches to publicly available sequences. The remaining 15 phylogroups have not been sequenced previously and may or may not represent novel taxa.

The identified phylogroups included (in alphabetical order): *R. acrifolia* Romagn., *R. aeruginea* Fr., *R. brevipes* Peck, *R. cessans* A. Pearson, *R. claroflava* Grove, *R. consobrina* (Fr) Fr., *R. cuprea* (Krombh.) J.E. Lange, *R. decolorans* (Fr.) Fr., *R. emetica* (Schaeff.) Pers., *R. exalbicans* (Pers.) Melzer et Zvára, *R. gracilis* Burl., *R. gracillima* Jul. Schäff., *R. integriformis* Sarnari, *R. lepidicolor* Romagn., *R. nauseosa* (Pers.) Fr., *R. nigricans* (Bull.) Fr., *R. pallescens* P. Karst., *R paludosa* Britzelm, *R. persicina* Krombh., *R. risigallina* (Batsch.) Sacc, *R. sanguinea* (Bull.) Fr., *R. sphagnophila* Kauffman, *R. turci* Bres., *R. versicolor* Jul. Schäff., *R. vesca* Fr., *R. xerampelina* (Schaeff.) Fr. and the sequestrate *Gymnomyces fallax* (Singer & A.H. Sm.) Trappe, T. Lebel & Castellano. There were two additional taxa that were most closely related to supported phylogroups that split up within OTUs: *R. nana* Killerm. (in OTU 25, phylogroup '*R. emetica* 2') and *R. nitida* (Pers.) Fr. (in OTU 18, phylogroup '*R. sphagnophila* 2'). In both cases, the sister phylogroups were found in different plant communities.

Comparing Russulaceae communities among vegetation types

Soil clone ITS sequences grouped into 42 nonsingleton 97% OTUs. MRPP analyses indicated that the overall distribution of *Russula* OTUs was not related to year (2004 vs 2005) or to soil horizon (organic vs mineral). We therefore pooled OTU counts across years and horizons within each site for subsequent ordinations. Autopilot mode in PC-Ord resulted in the selection of a three-dimensional solution in the NMS ordination of Sorenson distances among sites, with a final stress of 32.84 and instability of 0.098 after 500 iterations. The first axis represented 44% of the variation, whereas the three axes together accounted for 80% of the variation. Successional stage was strongly related to axis 1 (Pearson–Kendall correlation $r^2 = 0.535$), with a concomitant positive correlation with conifer abundance ($r^2 = 0.296$) and negative correlation with aspen abundance ($r^2 = 0.159$). As can be seen in Fig. 3, the black spruce sites are clustered on the right of the ordination, with the early-stage deciduous sites on the left. Late-successional upland sites dominated by white spruce occupy an intermediate position.

As a result of the transition from deciduous to coniferous species through succession at these sites, the influences of successional stage *per se* cannot be disentangled from the influences of host species on overall *Russula* community structure. Indeed, an MRPP analysis with years and horizons pooled, comparing sites with and without *Picea*, revealed a strong difference in the composition of these communities (effect size, $A = 0.052$, $P = 0.0003$). MRPP results were nonsignificant for the presence vs absence of other tree genera.

The black spruce sites are mostly in lowland, boggy areas and are spread over 100 km in Interior Alaska, and so would be expected to differ greatly in fungal species composition from the upland UP sites, all of which are within a few kilometers of one another within the Bonanza Creek watershed. We therefore carried out an additional ordination from which the black spruce sites were excluded, which again revealed a strong correlation between successional stage and *Russula* community structure (Fig. 4). Hence, the successional patterns are not an artifact of comparing sites with radically different soils, topographies or climate.

The results of the indicator species analyses contrasted somewhat with the MRPP analyses of overall community structure. The frequency and abundance of certain *Russula* OTUs were strongly related to soil horizon (Table 2). For example, OTU 7 (= *R. aeruginea*) was found predominantly in the mineral horizon, with an indicator value of 53.2, $P = 0.0054$. The majority of OTUs, however, had no strong horizon preference, in agreement with the MRPP analyses. We also found that particular *Russula* OTUs were indicators for the presence of *Betula*, *Populus* or *Picea* at the site. At a significance threshold of $\alpha = 0.05$, 15 of 42 OTUs were indicators for *Picea*, 13 for *Betula* and 13 for *Populus*. Because of the intercorrelations of tree occurrences at these sites, many of the same OTUs are indicators for multiple tree species. For example, OTUs 8 and 9 (= *R. gracilis* and *R. brevipes*, respectively) are positive indicators for both *Betula* and *Populus* and negative indicators for *Picea*, suggesting strong preferences for early-successional deciduous forests. By contrast, OTU 2 (= unidentified *Russula* sp.) was the second most abundant taxon, and was found only in black spruce sites (Table 2). OTU 1 (= *Russula cf. cessans*) was the most abundant taxon in this study, was common in black spruce sites and was very abundant in one of the three white spruce sites, but was essentially absent from the early-successional deciduous sites. Not surprisingly, it was a strong positive indicator for *Picea* (indicator value = 61.2, $P = 0.018$).

Discussion

In recent years, DNA-based studies of soil fungal communities have provided valuable insights into the biodiversity and ecology of fungi, as well as evidence that there is a large component of fungal diversity in soils that is still unknown (Schadt *et al.*, 2003; O'Brien *et al.*, 2005; Lynch & Thorn, 2006; Porter *et al.*, 2008; Buée *et al.*, 2009). Large-scale projects – including our own, of which the study presented here is a part – have immense potential to augment our current knowledge of fungal diversity. This study is the first to assess the phylogenetic diversity and habitat partitioning of

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Russula, one of the most diverse ECM genera, in the boreal forests of Interior Alaska.

Based on the phylogenetic breadth of our sequences, most, if not all, major phylogenetic clades of *Russula*, as defined in Miller *et al.* (2006), are represented in Alaska. This is comparable with the patterns seen in the ECM genus *Lactarius* (Geml *et al.*, 2009), but is in sharp contrast with the trend seen in the nonmycorrhizal saprotrophic *Agaricus* (Geml *et al.*, 2008), the only two other Alaskan genera for which similar biodiversity assessments are available. Some of the Alaskan phylogroups of *Russula* clearly matched known species, but many groups were unique, previously unsequenced, and with or without known close relatives. These taxa of unknown identity may or may not represent newly discovered species, the formal description of which is beyond the scope of this paper. When comparing the number of phylogroups detected by different sampling methods, we observed that 15 (30%) and 45 (90%) of the 50 phylogroups were found in sporocarp and soil clone sequences, respectively. Five groups (10%) were represented only by sporocarps, 35 (70%) only by soil clones and 10 (20%) by both (Fig. 2). This not only underlines the importance of combined sporocarp and soil sampling for biodiversity assessments in fungi, but also suggests that the true diversity of boreal *Russula* in Alaska may be higher than observed. The species accumulation curves and bootstrap estimates of species richness suggest that we have found the vast majority of the *Russula* species that were present in the sampled soils on the sampling dates. Nonetheless, it is very likely that additional species will be found if more soil samples are taken.

Russula species, using 97% OTUs as a proxy, showed strong habitat preference not only to one of the two major forest types, i.e. black spruce vs upland birch–aspen–white spruce forests, but often to vegetation successional stages as well. One important ecological difference between the two major vegetation types is that nearly all sampled black spruce sites are underlain by permafrost, whereas the birch–aspen–white spruce sites are not. At the landscape scale in the boreal region in general, the presence of permafrost strongly influences vegetation patterns (Camill, 1999), which, in turn, probably shape ECM communities, as suggested by our results.

One of the most striking findings by our study is the nearly three-fold difference in *Russula* species diversity between black spruce and mixed upland forests. Although black spruce forest sites generally harbored 6–11 taxa, there were 22–24 taxa found in upland forest sites. For comparison, diversity estimates for *Lactarius*, another diverse genus closely related to *Russula*, indicated 3–7 and 6–8 taxa for the same black spruce and upland sites, respectively (Geml *et al.*, 2009). Another intriguing finding is that species diversity in early-successional forests, i.e. 25 yr after fire, was just as high or higher than in mid- and late-successional forests, i.e. *c.* 100 and 220 yr after fire, respectively. The comparably high diversity seen throughout succession does not support earlier views, wherein species richness of ECM fungi is expected to increase with succession (Bigg, 2000), or the three-stage model of Dighton & Mason (1985) in which species richness increases from young to medium-aged stands, and strongly decreases in old stands to a very low final level. In ordination space, the early successional UP1 sites were more scattered, meaning that their *Russula* communities were more variable, than in either the UP2 or UP3 sites (Fig. 4). Interestingly, the same diversity pattern was detected in other fungal groups as well (Taylor *et al.*, 2010). Although the mechanisms responsible for this pattern are unclear, we speculate that stochastic colonization after disturbance may lead to higher variability in early succession, with a gradual filtering of the community to the best-adapted species in late succession.

Despite the great community differences among black spruce and upland mixed forests, there were quite a few phylogroups that occurred in both major types. However, the vast majority of these were shared between black spruce and late-successional, predominantly white spruce, upland forests. For example, taxa with strong preference for *Picea*-dominated sites, i.e. lowland *P. mariana* forests and late-successional upland *P. glauca* sites, were (in the order of decreasing frequency): *R. cessans*, unidentified *Russula* (OTU 2), *R. decolorans*, unidentified *Russula* (OTU 16), *Gymnomyces fallax*, *R. nigricans*, *R. turci*, *R. nauseosa*, *R. sanguinea*. These taxa were absent in early-successional mixed forest plots, probably because white spruce usually becomes more dominant as the upland forest matures, which is also accompanied by an increase in the insulating moss cover (Chapin *et al.*, 2006). The resulting habitat may, in some respects (e.g. litter quality, soil temperature, moisture and pH), be more similar to the black spruce sites than to the early- and mid-successional, birch- and aspen-dominated upland forests. On the other hand, it is currently unclear how much of the successional pattern is actually driven by host preferences. Some taxa might prefer *Picea* (or conifers in general) as ECM host, regardless of the habitat, whereas some appear to be restricted to certain types of spruce communities (e.g. OTU 2 in black spruce). Available information on the host specificity for some of these taxa confirms the pattern of occurrence seen in our data. For example, *R. decolorans* and *R. nauseosa* are considered to be specific to Pinaceae (Molina *et al.*, 1992).

Taxa that were found in both conifer and deciduous stands include: *Russula* sp. N21, *R. aeruginea*, *R. consobrina* and *R. persicina* (although this species was a positive indicator of deciduous trees, it also occurred in several black spruce sites). It is not clear whether these taxa are associated with spruce or with deciduous woody plants in *Picea*-dominated stands. Although OTUs identified as *R. sphagnophila* and *R. emetica* also occurred in both lowland and upland

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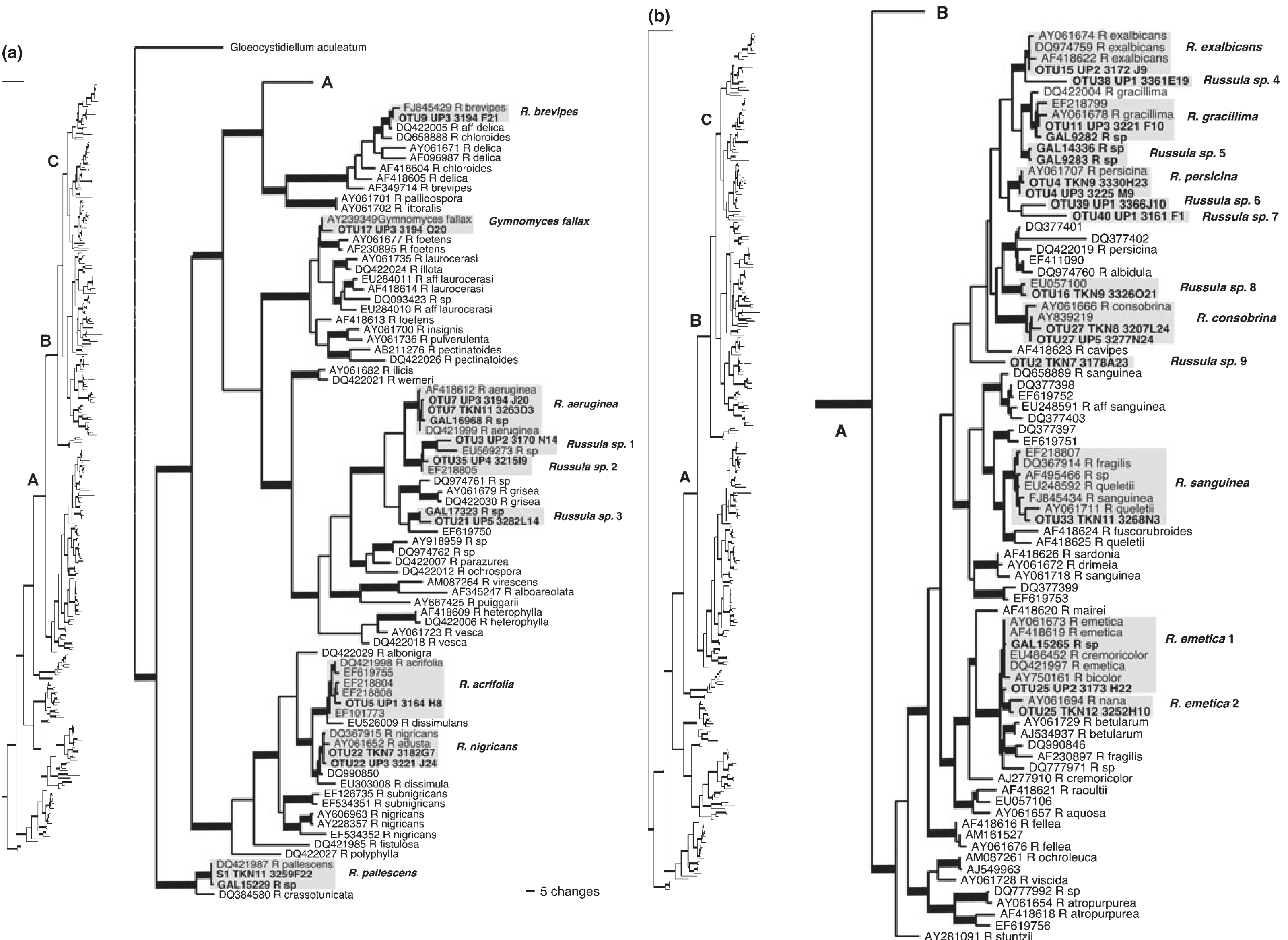

Fig. 2 Maximum-likelihood phylogram (Log$_e$ = 29 146.2106) inferred from the internal transcribed spacer (ITS) rDNA dataset showing the phylogenetic spread of boreal Alaskan *Russula* soil clone operational taxonomic units (OTUs) and sporocarp sequences (bold text) generated in this study among representatives of *Russula* taxa in GenBank. Sequences with GAL numbers were derived from herbarium specimens, whereas OTUs of UP and TKN sequences in bold are from soil clone libraries of upland mixed forest and lowland black spruce forest, respectively. Branches with Bayesian posterior probability support ≥ 0.95 are thickened. Phylogroups including Alaskan sequences are indicated by gray boxes and are named following OTU identification in Table 2 or based on closest relatives. GenBank sequences with no name attached are from unidentified environmental samples. Clades marked by 'A', 'B' and 'C' are shown in detail in parts (b), (c) and (d), respectively.

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Fig. 2 (Continued)

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Table 2 Distribution of *Russula* soil clones among habitats and soil horizons based on 97% internal transcribed spacer (ITS) sequence similarity operational taxonomic units (OTUs) and their taxon designation based on top BLAST match

OTU	Taxon designation	Indicator			Black spruce, acidic, dry						Black spruce, acidic, wet						Black spruce, nonacidic, dry						Black spruce, nonacidic, wet					
		Betula	*Populus*	*Picea*	TKN0001-M	TKN0001-O	TKN0122-M	TKN0122-O	TKN0012-M	TKN0012-O	TKN0109-M	TKN0109-O	TKN0015-M	TKN0015-O	TKN0022-M	TKN0022-O	TKN0123-M	TKN0123-O	TKN0126-M	TKN0126-O	TKN0039-M	TKN0039-O	TKN0119-M	TKN0119-O	TKN0040-M	TKN0040-O	TKN0051-M	TKN0051-O
1	*Russula cessans*			+	17	23	2	22								53	15	32	92	90	69	24	28	50	7	43	27	54
2	*Russula sp.*	-	-	+	13	3	37		48	5	223	24		5	45	3					16				36			
3	*Russula sp.*	+	+																									
4	*Russula persicina*	+	+		3									7					5	39	14			8				
5	*Russula acrifolia*		+	-																								
6	*Russula sp.* N21						8		21	5																		
7	*Russula aeruginea*				6	1	16		6		3						8								1			
8	*Russula gracilis*	+	+	-																								
9	*Russula brevipes*	+	+	-																								
10	*Russula risigallina*		+	-																								
11	*Russula gracillima*	+	+																									
12	*Russula sp.*		+	-																								
13	*Russula cf. vesca*	+																										
14	*Russula decolorans*				1		2			1	1			9	13										1			
15	*Russula_exalbicans*		+	-																								
16	*Russulaceae sp.*															1				43								
17	*Gymnomyces fallax*																											
18	*Russula sphagnophila*												5	9														
19	*Russula sp.*		+	-																								
20	*Russula integriformis*		+	-																								
21	*Russula sp.*		+	-																								
22	*Russula_nigricans*								11	3																		
23	*Russula_lepidicolor*	+																										
24	*Russula xerampelina*	+																										
25	*Russula emetica*							1			1		1															
26	*Russula cuprea*	+	+	-																								
27	*Russula consobrina*							5								1												
28	*Russula turci*								7																			
29	*Russula sp.*			-																								
30	*Russulaceae sp.*																											
31	*Russula sp.*	+																										
32	*Russula nauseosa*																											1
33	*Russula sanguinea*					1																1						1
34	*Russula versicolor*			-																								
35	*Russulaceae sp.*																											
36	*Russula sp.*																											
37	*Russula sp.*																											
38	*Russula sp.*																											
39	*Russula sp.*			-																								
40	*Russulaceae sp.*			-																								
41	*Russula sp.*																											
42	*Russula sp.*																											
	Number of OTUs per site and soil horizon				5	4	5	3	5	4	4	1	2	4	2	4	2	1	2	3	3	2	1	2	4	1	1	3
	Number of OTUs per habitat				Mineral: 8; Organic: 8 Total: 11						Mineral: 5; Organic: 7 Total: 9						Mineral: 4; Organic: 4 Total: 6						Mineral: 4; Organic: 4 Total: 7					

OTU	Taxon designation	Early successional upland						Mid-successional upland						Late successional upland						Grand Total
		UP1A-M	UP1A-O	UP1B-M	UP1B-O	UP1C-M	UP1C-O	UP2A-M	UP2A-O	UP2B-M	UP2B-O	UP2C-M	UP2C-O	UP3A-M	UP3A-O	UP3B-M	UP3B-O	UP3C-M	UP3C-O	
1	*Russula cessans*			2										288	22	3		7		960
2	*Russula sp.*																			558
3	*Russula sp.*			10	7	3	2	121	77	95	29	2	19				12			377
4	*Russula persicina*			5	174					2		9	5	61	6	8	2	5		353
5	*Russula acrifolia*					16	11	3	3	75		62	47				6	64		287
6	*Russula sp.* N21	23		1						37	1			39	2	13		50	5	205
7	*Russula aeruginea*	27		4	9			5		67	6			20		1	11	1		192
8	*Russula gracilis*		2	1	41	4		39	7	63	3	5	6	3	1	2	6	1	2	186
9	*Russula brevipes*	3	8	7	10	13	5	10		19		20	4	16		5		11		131
10	*Russula risigallina*					5	2			33		38	21			8			3	110
11	*Russula gracillima*	1		6	7	4	1	1	10	4	23		1		2		41		2	103
12	*Russula sp.*					12	6	24				36	14					1		93
13	*Russula cf. vesca*															18		54	6	78
14	*Russula decolorans*				1									2	4	4	1	15	14	69
15	*Russula_exalbicans*				10	4	3	9	5	9		4	2							46
16	*Russulaceae sp.*																			44
17	*Gymnomyces fallax*													39	1					40
18	*Russula sphagnophila*										4						4	8	8	38
19	*Russula sp.*					19	12					2						1		34
20	*Russula integriformis*					11	13					1	7							32
21	*Russula sp.*					9		7				2	7							25
22	*Russula_nigricans*															5	4			23
23	*Russula_lepidicolor*															9		3	7	19
24	*Russula xerampelina*								13	1	1									15
25	*Russula emetica*			2				1				2	2							10
26	*Russula cuprea*	8	1											1						10
27	*Russula consobrina*								3											9
28	*Russula turci*																			7
29	*Russula sp.*					3	2													5
30	*Russulaceae sp.*						4													4
31	*Russula sp.*														1	1		1	1	4
32	*Russula nauseosa*														2					3
33	*Russula sanguinea*																			3
34	*Russula versicolor*		1			1														2
35	*Russulaceae sp.*											2								2
36	*Russula sp.*														1	1				2
37	*Russula sp.*								1		1									2
38	*Russula sp.*					1					1									2
39	*Russula sp.*			1	1															2
40	*Russulaceae sp.*		1	1																2
41	*Russula sp.*									1		1								2
42	*Russula sp.*																	2		2
	Number of OTUs per site and soil horizon	5	5	11	9	14	11	10	8	14	9	14	12	9	10	13	9	15	9	
	Number of OTUs per habitat	Mineral: 22; Organic: 19 Total: 24						Mineral: 18; Organic: 20 Total: 22						Mineral: 20; Organic: 18 Total: 23						

OTUs represented by only a single clone sequence are not shown. M, mineral soil horizon; O, organic soil horizon. OTUs with significant indicator status are marked as either positive (+) or negative (–) indicators of the major boreal tree genera (cells with nonsignificant values are blank).

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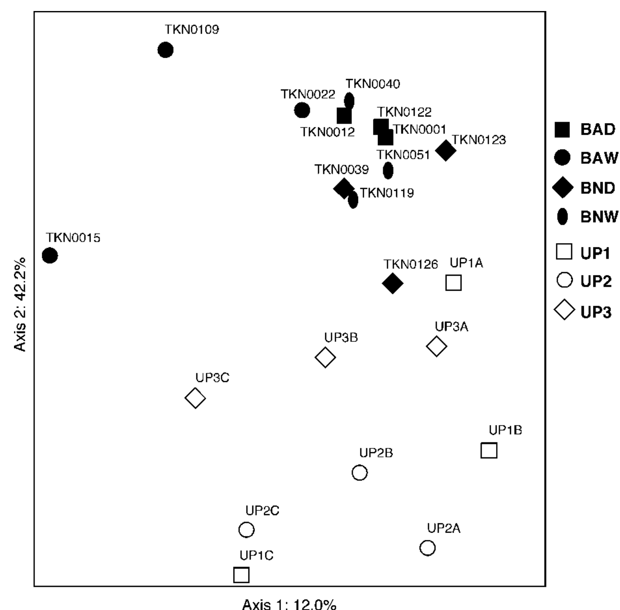

Fig. 3 Nonmetric multidimensional scaling (NMS) ordination plot for *Russula* communities from various black spruce (*Picea mariana*) and upland birch–aspen–white spruce (*Betula neoalaskana–Populus tremuloides–Picea glauca*) forest types. BAD, black spruce, acidic, dry; BAW, black spruce, acidic, wet; BND, black spruce, nonacidic, dry; BNW, black spruce, nonacidic, wet; UP1, early-successional upland birch–aspen–white spruce; UP2, mid-successional upland birch–aspen–white spruce; UP3, late-successional upland birch–aspen–white spruce.

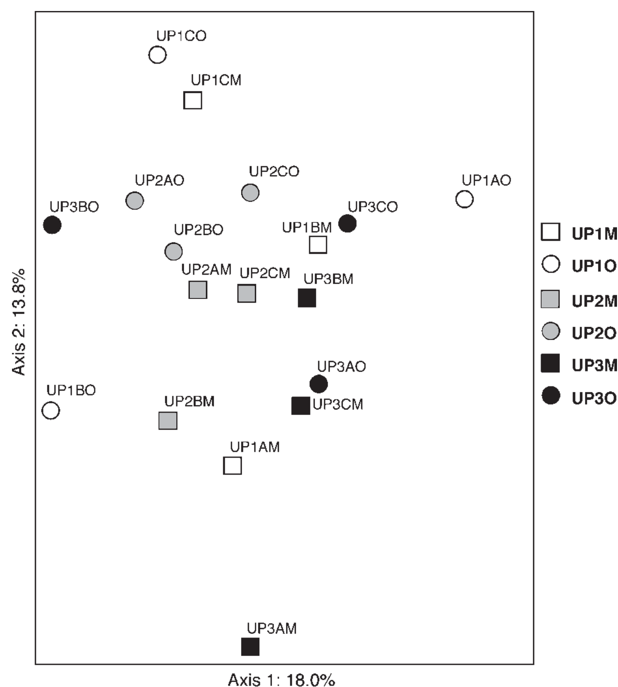

Fig. 4 Nonmetric multidimensional scaling (NMS) ordination plot for *Russula* communities from various soil horizons and successional stages of upland birch–aspen–white spruce forests (*Betula neoalaskana–Populus tremuloides–Picea glauca*). UP1, early-successional upland birch–aspen–white spruce; UP2, mid-successional upland birch–aspen–white spruce; UP3, late-successional upland birch–aspen–white spruce; M, mineral soil horizon; O, organic soil horizon.

sites, these OTUs were split into two phylogroups that showed contrasting habitat preferences. In the *R. sphagnophila* complex, sequences from upland sites grouped with *R. sphagnophila*, whereas sequences from black spruce stands grouped with *R. nitida*. Similarly, in the *R. emetica* complex, soil clones from mixed deciduous–conifer forests grouped with *R. emetica*, and sequences from black spruce communities grouped with *R. nana*. The occurrence of *R. nana* in black spruce may seem surprising at first, because this taxon is considered as an arctic–alpine specialist, usually found above the treeline. However, because of their cold microclimates, limited nutrients and open canopy, black spruce muskegs can sustain several predominantly arctic–alpine plant species, such as *Betula nana* L., *Rubus chamaemorus* L., *Carex bigelowii* Torr. etc. (Hollingsworth *et al.*, 2006), and *R. nana* has been reported to be associated with *B. nana* in Alaska (Miller, 1982).

There were many more species that were restricted to upland sites and were positive indicators of deciduous trees (birch and aspen), and often negative indicators of conifers, e.g. unidentified *Russula* (OTU 3), *R. acrifolia*, *R. gracilis*, *R. brevipes*, *R. risigallina*, *R. gracillima*, unidentified *Russula* (OTU 12), *R. cf. vesca*, *R. exalbicans*, unidentified *Russula* (OTU 19), *R. integriformis*, unidentified *Russula* (OTU 21), *R. lepidicolor*, *R. xerampelina*, *R. cuprea* and unidentified *Russula* (OTU 31). Overall, similarly to that observed in *Lactarius* (Geml *et al.*, 2009), our results show that some of the diversity is niche based, especially along a host and possibly successional axis, because most OTUs predictably occurred in specific habitats, regardless of geographical location.

In general, preference for soil horizon was much less pronounced than preference for habitat. Nonetheless, there were some taxa that tended to be associated with the mineral horizon, e.g. *Russula* sp. N21 (OTU 6), *R. aeruginea*, *R. gracilis*, *R. brevipes*, *R. risigallina*, unidentified *Russula* (OTU 12) and *Gymnomyces fallax*, whereas others predominantly occurred in the organic horizon, such as *R. persicina* and *R. gracillima*. It should be noted, however, that only three were significant or marginally significant indicators for soil horizon, all for the mineral horizon: *Russula* sp. N21 ($P = 0.034$), *R. aeruginea* ($P = 0.005$) and *R. brevipes* ($P = 0.055$). When comparing the soil horizons in terms of species diversity, mineral soils harbored 1–5 taxa in black spruce and 5–15 in upland forests, whereas we detected 1–4 OTUs in black spruce and 5–14 in upland organic soils (Table 2). Although these ranges may not suggest a significant difference at first glance, paired *t*-test showed that the mineral horizon generally contained significantly more OTUs at a given site than did the organic horizon ($P = 0.0179$).

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As a response to recent warming, the boreal forest is on the brink of significant changes in both composition and function. Projections suggest that warming-induced drought stress and decreased fire interval will result in the expansion of deciduous forests at the expense of conifers, particularly white spruce (Calef *et al.*, 2005; Chapin *et al.*, 2006). Such changes will undoubtedly alter ECM community dynamics and species abundance, but the nature of this response is difficult to predict given our current knowledge. Therefore, our results not only provide the first assessment of the biodiversity of *Russula* and its partitioning among different habitats in Alaska, but also serve as a legacy dataset against which to evaluate changes in fungal community structure that are likely to occur under continuing climate and vegetation change.

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